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Breast cancer is a multi-step genetic process involving mutations of oncogenes and tumor suppressor genes. With a few notable exceptions the genetic determinants that contribute to the development of breast cancer remain unknown. We have developed and applied a novel retrovirus-based library screening strategy, coupled to a biological assay for growth transformation, to identify novel oncogenes in breast cancer development. The approach involves the isolation of mRNA from noninvasive (MCF-7) and invasive (BT549 and Hs578T) human breast carcinoma cell lines to generate full length cDNA sequences, and introduced into a retrovirus expression vector. The retrovirus, encoding genes expressed in breast cancers, is infected into Rat-1 fibroblasts or RIE-1 rat intestinal epithelial cells and screened for the appearance of foci of transformed cells. The resulting transformed cells are isolated and the retrovirus-associated cDNA is rescued. After sequence analyses, and verification of transforming activity of the isolated sequences, further analyses are done to assess the contribution of the isolated genes to breast cancer development. From these screens, we have identified the FGF receptor 2 and the Raf-1 serine/threonine kinase.

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## INTRODUCTION

Our laboratory has refined and applied a novel retrovirus-based library screening strategy (Whitehead et al., 1995a; Whitehead and Khosravi-Far, 99 A.D.; Whitehead et al., 1995b; Tognon et al., 1998), coupled to a biological assay for growth transformation, to identify novel oncogenes in cancer cell lines. Importantly, recently our lab has extended this approach, using different biological assays, to identify oncogenes in patient-derived human acute myeloid leukemias (Reuther et al., 2000; Reuther et al., 2002). The success of our previous and recent applications of a functional screen to identify novel genetic elements in cancer stimulated our interest to apply this approach to dissect the genetic basis of breast cancer.

The approach that I have taken involves the isolation of mRNA from noninvasive (MCF-7) and invasive (BT549 and Hs578T) human breast carcinoma cell lines to generate full length cDNA sequences, and introduction of those sequences into a retrovirus expression vector (Figure 1). The retrovirus, encoding genes expressed in breast cancers, is then infected into Rat-1 fibroblasts or RIE-1 rat intestinal epithelial cells and screened for the appearance of foci of transformed cells (Reuther et al., 2000; Reuther et al., 2002). The resulting transformed cells are isolated and the retrovirus-associated cDNA is rescued. After sequence analyses, and verification of transforming activity of the isolated sequences, further analyses are done to assess the contribution of the isolated genes to breast cancer development. From these screens, we have identified the FGF receptor 2 (FGFR2) and the Raf-1 serine/threonine kinase (Figure 1). There is already evidence for the importance of FGFR2 overexpression in breast cancer development (Heiskanen et al., 2001; Tannheimer et al., 2000). The identification of Raf-1 is especially intriguing in light of the recent identification of mutated Raf in human cancers, in particular melanomas and colon cancers (Davies et al., 2002; Rajagopalan et al., 2002). Thus, the potential relevance of these identified genes to breast cancer development help validate the retrovirus screening technique as a potentially valuable and powerful approach to identify aberrant oncogene activation in breast cancer.

These studies accomplished two main goals. First, since the majority of the assays and techniques utilized in the study were new to me, the study was an excellent vehicle to extend my expertise in recombinant DNA techniques and also to develop an understanding and appreciation of breast cancer cell biology and oncogenesis. Second, an analysis of some of the transforming genes identified several interesting signaling proteins that I will continue to analyze. These long-term analyses will further strengthen my understanding of breast cancer and approaches for studying the genetics of breast cancer.

## BODY

This fellowship was awarded originally to another postdoctoral fellow in the lab (Dr. Janiel Shields). When she obtained a DOD grant to pursue the study

of neurofibromatosis and Ras regulation, this fellowship was transferred to me to continue the proposed studies. Therefore, I was involved in this project for a little more than a year. Additionally, since this project was not one of the directions of my studies at the time I was put on the fellowship, this research direction was a completely new area of study for me. Therefore, the first part of my involvement in this project was to first learn how to generate the retrovirus cDNA expression library. Since the previous work had already generated a library representing mRNA from MCF-7 cells, I chose to make a library from a second, invasive breast carcinoma cell line (Hs578T).

My initial efforts at making the retrovirus cDNA expression library proved to be fairly successful. Although what was made did not fully represent all possible genes expressed in Hs578T cells, I decided to proceed with this library to gain experience in the transformation screening. For these screens, I used Rat-1 rat fibroblasts and RIE-1 rat intestinal epithelial cells. The original proposal proposed to use MCF-10A human breast epithelial cells (Soule et al., 1990; Basolo et al., 1991), which have been shown to be sensitive to transformation by activated Ras or HER2. These cells have properties of normal cells, do not grow in soft agar, or form tumors in nude mice. However, we had extreme difficulty with these cells; this involved mainly their tendency to drift with a very short time of subculturing and tended to become partially-transformed. After receiving additional stocks of MCF-10A, and after being advised that how they were subcultured was critical for them to maintain normality, we still found that the line drifted. Hence, I returned to Rat-1 and RIE-1 cells for my screens. Both of these cell lines have normal growth characteristics and never undergo spontaneous transformation in cell culture (Oldham et al., 1996). Thus, if I got a transformation hit on these cells, it would likely be a real event, and hence, worth the effort to subclone out the transforming gene.

For my transformation screens, I used a retrovirus vector that contained an activated H-Ras(61L) allele as a positive control and the empty retrovirus vector as a negative control. Whereas cells infected with the empty vector never showed any focus forming activity, the Ras vector readily caused transformation. By comparison, the breast library retrovirus generated approximately 12 foci of transformed cells. This suggested that my assay conditions were appropriate and that my library contained transforming genes.

The next step in the process was to use PCR-mediated gene amplification to isolate the retrovirus-associated sequences from the transformed cell populations. When the sequences were isolated and subcloned, then subjected to DNA sequencing, the disappointing result was the isolation of sequences encoding activated H-Ras(61L). I subcloned out this sequence from over 10 separately isolated transformed foci. Unfortunately, this meant that the transforming activity seen with my library represented a contamination from my positive control H-Ras(61L) retrovirus.

Therefore, I have repeated the generation of the library from the beginning. I have isolated new RNA from the breast line and have generated a new retrovirus cDNA expression library, which represents many more genes than the original library. New procedures were implemented to prevent contamination

of the library or screening assay with control retrovirus. Consistent with the better representation of potential novel breast cancer oncogenes in the new library, the new transformation screens in Rat-1 and RIE-1 cells have so far yielded 30-40 foci, whose characterization is currently underway.

The other aspect of my work has been the characterization of two transformants that we had isolated from a separate screen of the MCF-7 library. This involved the fibroblast growth factor receptor 2 (FGRFR2) and the Raf-1 serine/threonine protein kinase (Figure 1). When these cDNA sequences were isolated and subjected to sequence analyses, we were surprised to find that they encoded wild type sequences for both proteins. Transformation assays done on these isolated genes verified their potent transforming activity. Thus, transformation caused by these two genes is a consequence of gene overexpression. Therefore, the next step in these studies will be to determine if their overexpresion can cause transformation of human breast epithelial cells. We will use telomerase-immortalized human mammary epithelial cells (HMECs) for these studies (Elenbaas et al., 2001). The other step in these studies is to determine if either gene is overexpressed in breast carcinoma cell lines, and if so, whether downregulation, for example via RNAi, would revert the transformed pheonotype of these cells. These two approaches will help verify or validate the importance of our isolated genes in breast cancer.

## KEY RESEARCH ACCOMPLISHMENTS

- Generation of pCTV3 retrovirus cDNA expression libraries representing genes expressed in invasive Hs578T breast carcinoma cells.
- Expression screening using Rat-1 and RIE-1 focus formation assays for transforming genes.
- PCR isolation of transforming genes from transformed colonies identified in the library screen.
- The isolation of two sequences encoding wild type FGFR2 and Raf-1 and the verification that their overexpression can cause transformation of Rat-1 and RIE-1 cells.

## REPORTABLE OUTCOMES

Abstract Poster presentation at the DOD Era of Hope, September 25-28, 2002, Orlando, FL

Fiordalisi, J.J., Lambert, Q.T., Cox, A.D. and Der, C.J. Aberrant signaling in breast cancer development.

## CONCLUSIONS

Since my involvement in this project was relatively short-term, and I encountered a contamination problem in my library screen, no significant scientific conclusions were reached from my studies. My accomplishments in these studies were mainly technical. New approaches that I gained from this work included the generation of retrovirus cDNA expression libraries and the use of cell lines to perform expression screening and selection for transforming genes. Additionally, I had significant initial difficulty with the PCR techniques used to rescue cDNA sequences from the transformed populations. Thus, in trouble-shooting this step in the analyses, I greatly strengthened my skills in recombinant DNA work. Finally, this project allowed me to work with breast cancer cell lines and to acquire a strong foundation in breast cancer cell biology. Thus, while the funding for this project has been completed, I plan on continuing my studies to search for novel oncogenes in breast cancer.

A second research direction that is a direct outcome of these studies is my use of microarray analyses to study the gene expression changes caused by the treatment of Ras-transformed HMECs with various pharmacologic inhibitors of Ras, in particular, farnesyltransferase inhibitors. In my future studies, I will use HMECs as one cell system to study the importance of my identified oncogenes in breast cancer. For example, will my identified oncogenes promote the growth transformation of HMECs that have been immortalized by hTERT and SV40 largeT/small t expression? If so, I will also incorporate these cells into my microarray studies to evaluate gene expression changes associated with breast epithelial cell transformation by different oncogenes.

I have two ideas for my future library screens (Figure 1). For my future screens, I would also like to utilize HMECs, rather than Rat-1 or RIE-1 cells. This would provide a better match for the cell type from which the cDNA libraries were made. It is highly conceivable that oncogenes active in breast cancer may not be active and detectable in other cell types. Hence, the use of nontransformed human breast epithelial cells should add further strength to this screening effort. A second direction would be to use MCF-7 cells as a screen for invasion-inducing genes. MCF-7 breast carcinoma cells are noninvasive and show essentially no invasion activity in Matrigel invasion assays in vitro. Hence, they provide a nice biological selection scheme to isolate genes expressed in the invasive Hs578T cells that can promote the invasion of MCF-7 cells.

In summary, if one important goal of this fellowship support was to encourage new junior level investigators to pursue a career in breast cancer research, this has been achieved for me. I am excited about continuing these and other studies on breast epithelial cell oncogenesis and I hope to identify genes that can then be used as a foundation for my future research as an independent researcher.

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**APPENDICES** – Figure 1.

Figure 1. Expression Cloning of Novel Oncogenes in Breast Cancer